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Duration of immunity induced in chickens by an attenuated live *Salmonella enteritidis* vaccine and an inactivated *Salmonella enteritidis/typhimurium* vaccine

Dauer der Immunität nach Impfung von Hühnern mit einer Salmonella enteritidis Lebend- und einer Salmonella enteritidis/typhimurium Inaktivatvakzine

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Summary

The aim of this study was to examine the duration of immunity of different vaccination schemes using the *S. enteritidis* live vaccine Gallivac® Se and the *S. enteritidis-S. typhimurium* inactivated vaccine Gallimune® Se+St. Three groups of Lohman Brown chickens were used. Group one was vaccinated three times orally with Gallivac® Se at weeks one, seven and 13 of age. Group two was vaccinated twice orally with Gallivac® Se in weeks one and seven and once i.m. with Gallimune® Se+St in week 14 of age. A third group was not vaccinated and served as the control group. Eight randomly selected chickens from each of the three groups were challenged with a nalidixic acid resistant *S. enteritidis* PT4 strain in weeks 24, 51 and 71 of age and the same number of animals were challenged with a *S. typhimurium* DT 104 strain in weeks 26, 54 and 73 (75) of age. The chickens were euthanised seven days post challenge and the number of challenge strain organisms (log_{10} cfu) in the liver and on caecal mucosa was determined. The quantitative investigation of the challenge strain in the liver and caecal mucosa revealed a statistically significant ($p < 0.05$) lower challenge strain burden in the vaccinated groups compared with the non-vaccinated control group up to week 71 (73) of age. The protective effects were demonstrated for both challenge strains.

Keywords: chicken, Live Salmonella vaccine, Salmonella, zoonosis

Zusammenfassung

Das Ziel der vorliegenden Arbeit bestand in der Untersuchung der Dauer der Immunität nach Anwendung verschiedener Impfschemata unter Verwendung der *S. enteritidis* Lebendvakzine Gallivac® Se und der *S. enteritidis-S. typhimurium* Inaktivatvakzine Gallimune® Se+St. Dazu wurden Hühner (Lohman Brown) dreimal oral in der ersten, siebten und 13. Lebenswoche mit der Lebendvakzine Gallivac® Se geimpft (Gruppe 1). Tiere der Gruppe 2 erhielten die Lebendvakzine zweimal oral in der ersten und siebten Woche und die Inaktivatvakzine Gallimune® Se+St einmal i. m. in der 14. Lebenswoche verabreicht. Die Tiere der Gruppe 3 wurden nicht vakziniert und dienten als Kontrolle. In der 24., 51. und 71. Lebenswoche wurden jeweils 8 zufällig ausgewählte Tiere pro Gruppe einer Infektionsbelastung mit einem nalidixinsäureresistenten *S. enteritidis*-PT4-Stamm und die jeweils gleiche Anzahl von Hühnern in der 26., 54. und 73. (75.) Lebenswoche mit einem nalidixinsäureresistenten *S. typhimurium*-DT 104-Stamm unterzogen. Am siebten Tag nach der Infektion wurden die Tiere schmerzlos getötet und der Infektionsstammgehalt (log_{10} cfu) in der Leber und Blinddarmschleimhaut bestimmt. Es traten signifikante Unterschiede ($p < 0,05$) in der Infektionsstammbesiedlung zwischen beiden geimpften Gruppen und der Kontrollgruppe bis zur 71. (73.) Lebenswoche auf. Schutzeffekte konnten für beide Infektionsstämme, *S. enteritidis* und *S. typhimurium*, nachgewiesen werden.

Schlüsselwörter: Huhn, Salmonella Lebendimpfstoff, Salmonella, Zoonose

Introduction

Salmonella spp. are one of the major causes of food-borne illnesses in humans. The most important source is poultry derived food, mainly eggs and egg-products but also chicken meat (Rodrigue et al., 1990). In the European Union *S. enteritidis* and *S. typhimurium* are among the most frequently isolated serovars from farms with laying hens (Anonymous, 2010). Both serovars play an important role in salmonellosis in humans (Anonymous, 2009a). Improved management, including better biosecurity, cleaning and disinfection and rodent control combined with vaccination are implemented to reduce the prevalence of salmonellae on farms to meet one of the demands of the European Regulation No. 2160/2003 (Anonymous, 2003). Within the European Union, two live vaccines are currently registered based on *S. enteritidis*, two on *S. typhimurium*, and one vaccine based on *S. gallinarum*. In addition, a series of inactivated vaccines that contain *S. enteritidis* and *S. typhimurium* are authorised.

Since salmonellae have the capability to invade cells and replicate intracellularly (Suter 1956), great emphasis is put on orally applied live vaccines due to their capacity of inducing local, humoral and particularly cell mediated immune mechanisms (Lehmann et al., 2006; Carvajal et al., 2008). Few studies have been carried out regarding the combined use of live and inactivated Salmonella vaccines over the entire laying period (Hafez et al., 2001).

Animal models are often used to assess the efficacy of salmonella vaccines. The basis of these models is the investigation of the excretion and persistence after infection with a virulent strain (Anonymous, 2009b). In addition models have proved that efficacy can be evaluated by comparing the colonisation of the inner organs after oral challenge with antibiotic resistant salmonella strains (Cooper et al., 1994; Methner et al., 1995; Hassan and Curtiss 1996). The so called "seeder bird method" (Bolder et al., 1992, Cameron and Carter, 1992) is the closest to the natural route of infection. For this purpose chicks will be infected with a defined dose of a Salmonella challenge strain representing the "seeder birds". Subsequently animals of a vaccinated and a control group will be exposed to the challenge strain via these "seeder birds" which are placed amidst them. At last, the colonisation of organs with salmonella can be evaluated and compared between the vaccinated and control groups. The aim of our investigations was to test the duration of immunity of two vaccination schemes using the live vaccine Gallivac® Se and the inactivated vaccine Gallimune® Se+St. The evaluation was carried out by comparing the colonisation of the challenge strain in the liver and caecum mucosa up to the end of the laying period after an oral challenge with a nalidixic acid resistant *S. enteritidis* and *S. typhimurium* strain.

Material and Methods

Chickens

Altogether 600 commercial *Salmonella* spp. free Lohmann Brown chickens were used. During the rearing and laying period, three groups with 200 chickens each were kept in isolation units on deep litter and a standardised light regime was applied. The birds were fed according to their age with a commercial feed for chicks, young hens or layers. Drinking water was available ad libitum. At least once a month samples of feed, dust, faeces and boot swabs from each isolation unit were examined bacteriologically for salmonellae. From those animals that had been vaccinated with Gallivac® Se the vaccine strain was re-isolated for a short period of time post vaccination on "sock swabs" as expected. Furthermore serum samples from the vaccinated birds and from the control birds were examined for antibodies against *S. enteritidis* and *S. typhimurium* before challenge.

Vaccines

Two groups of chickens were vaccinated with the minimal effective dose (1×10^8 cfu/animal) of the vaccine Gallivac® Se (Merial, France). The vaccine is based on the strain *Salmonella enteritidis* 441/014 (adenine-histidine auxotroph) and is also registered in Germany and Hungary under the name "Salmovac SE" (IDT Biologika GmbH, Germany). For the booster vaccination (group 2) the *S. enteritidis*/*typhimurium* combination vaccine Gallimune® Se+St (Merial, France) was used.

Challenge strains

Spontaneously occurring nalidixic acid resistant strains of *S. enteritidis* PT4 (strain 147 N) and *S. typhimurium* DT 104 (strain 27 N) were used for the challenge infection (The strains were kindly provided by Dr U. Methner, Institute of Bacterial Infections and Zoonoses" at the Friedrich Loeffler Institute, Jena). Both strains had been isolated from the vitellus of hens' eggs and have been used as challenge strains in several studies before (Methner et al., 2001; Methner et al., 2004; Springer et al., 2006; Carvajal et al., 2008). Cultivation of the challenge strains was carried out over two pre-cultures in a Tryptone Soya Broth containing medium (*S. typhimurium* 6/83 Medium, IDT Biologika GmbH). After cultivation the strains were washed, concentrated and stored in PBS (pH 7.2) at –20°C until use.

Experimental design and microbiology

Table 1 shows the allocation of the groups. Chickens of group 1 were vaccinated by oral gavage on the second day of age and in their 7th and 13th week of age with the *S. enteritidis* live vaccine Gallivac® Se. Chickens of group 2 were vaccinated orally with the live vaccine on the second day and in the 7th week of age. Chickens of this group were re-vaccinated with a single intramuscular dose of Gallimune® Se+St in the 14th week of age. Chickens of group

TABLE 1: *Allocation of the groups and dates of the treatment*

Group	n	Vaccination with Gallivac® Se	Vaccination with Gallimune® Se+St	Challenge dates (age)
1	200	1st, 7th, 13th week of age	None	24th, 51st, 71st week of age (*S. enteritidis*) 26th, 54th, 73rd, 75th week of age (*S. typhimurium*)
2	200	1st, 7th week of age	14th week of age	24th, 51st, 71st week of age (*S. enteritidis*) 26th, 54th, 73rd, 75th week of age (*S. typhimurium*)
3	200	None	None	24th, 51st, 71st week of age (*S. enteritidis*) 26th, 54th, 73rd, 75th week of age (*S. typhimurium*)

TABLE 2: *Results of the challenge studies with strain Salmonella enteritidis 147 N, measured seven days post challenge*

Group	n	Time of Challenge (Week of life)	Challenge strain content (mean ± SD) in log cfu/g Liver	Caeca
1	8	24	1.83 ± 0.33[1]	5.47 ± 0.32[1]
2	8	24	1.36 ± 0.74[1]	5.14 ± 0.72[1]
3	8	24	2.54 ± 0.41	6.28 ± 0.36
1	8	51	1.55 ± 0.37[1]	5.29 ± 0.66[1]
2	8	51	1.40 ± 0.72[1]	4.72 ± 1.15[1]
3	8	51	2.72 ± 0.46	7.33 ± 1.34
1	8	71	1.29 ± 0.64[1]	4.88 ± 1.19[1]
2	8	71	2.04 ± 0.39[1]	5.19 ± 0.64[1]
3	8	71	2.51 ± 0.35	6.62 ± 1.30

Significant difference between the vaccinated group and the control group (Mann Whitney U test, one-tailed test). [1] significance level $p < 0.05$.

3 served as controls and were not vaccinated. In the 24th, 51st and 71st week of life eight randomly selected chickens per group were placed in separate isolation units. Blood samples were collected for serological examination. Subsequently, the birds were challenged by oral gavage with *S. enteritidis* 147 N. A further eight chickens per group were also placed in separate isolation units in weeks 26, 54, 73 and 75. After taking blood samples these chickens were challenged orally with *S. typhimurium* 27N. The challenge doses for strains were 5 x 10^8 and 1 x 10^9 cfu per animal, respectively. Seven days post challenge the chickens were euthanased. Bacterial counts of *S. enteritidis* 147 N and *S. typhimurium* 27 N in liver and caecal mucosa were examined using a standard plating method as described by Methner, et al., 1995. Caeca were first cleared from faeces, then the caecal mucosa was scraped with a sterile slide. The mucosa was weighed, diluted 1:3 with PBS (pH 7.2) and homogenised. Then serial ten-fold dilutions of the homogenised organ samples were prepared in PBS and plated onto desoxycholate citrate agar (HEIPHA) supplemented with sodium nalidixate (50 µg/ml). Plates were incubated at 37°C for 18–24 hours.

Serum samples from the vaccinated and control birds were examined for antibodies against *S. enteritidis* and *S. typhimurium* prior to the challenges using the FLOCKSCREEN® *S. enteritidis* and *S. typhimurium* ELISA kits (X-OVO LIMITED, United Kingdom).

Each individual challenge test was carried out as a controlled test. The control groups were not vaccinated. Allocation of the birds to the groups was random. A minimum of eight birds per group was planned in order to show the significant difference between vaccinated and non-vaccinated chickens. In cases where the difference between vaccinated and non vaccinated groups could not be classified as significant due to high variance within the groups the study protocol allowed for a second examination of eight chickens per group to be carried out in the same manner and to run a combined statistical evaluation of that data.

Statistical analysis

The viable bacteria counts and antibody values were converted into logarithmic form. For the purpose of statistical analysis, a log10 viable count of < 1.47 (limit for direct plating detection) obtained from samples becoming positive only after enrichment was rated as log10 = 1.0. A sample which yielded no Salmonella growth after enrichment was rated as log10 = 0. The Mann and Whitney test was used for the statistical analysis of the differences between the vaccinated groups and the control groups (SPSS® 7.5 for WINDOWS and R 2.12.0).

Results

Vaccination of chickens with the *S. enteritidis* live vaccine and the inactivated *S. enteritidis*/*typhimurium* combined vaccine (group 2) raised significantly higher pre-challenge antibody titres against antigens of *S. enteritidis* and *S. typhimurium* at the start, in the middle and at the end of the laying period compared to the vaccination with the live vaccine for three times (group 1) and to non vaccinated control animals (Fig. 1, 2).

After oral challenge of the birds with the *S. enteritidis* strain 147 N in the 24th, 51st and 71st week of age, significant differences ($p < 0.05$) in the colonisation of the liver and caecal mucosa with the challenge strain were shown between each vaccinated group (groups 1 and 2) and the control group (Tab. 2).

Table 3 shows the results of the examination of the protective effects of the vaccination towards challenge of the groups with the strain *S. typhimurium* 27 N at different time points during the laying period. Due to a high variance of the colonisation of the caecal mucosa with *S. typhimurium*, the differences between groups 2 and 3 following the challenge at 26 weeks and also the differences between groups 1 and 3 following the challenge at 54 weeks were not significant ($p > 0.05$). At the end of the laying period (73rd week of age) significant differences in the *S. typhimurium* challenge strain content in liver and caecal mucosa could be determined between the group vaccinated with both the live *S. enteritidis* and inactivated *S. enteritidis*/*typhimurium* vaccines (group 2) and the non vaccinated control group. As for the *S. typhimurium* challenge a significant difference in challenge strain content was seen for caecal mucosa ($p < 0.05$) between the

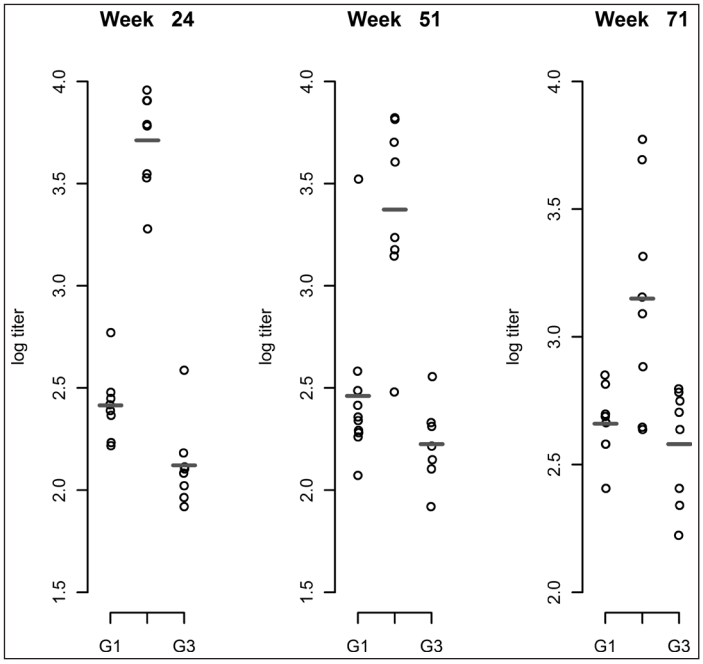

FIGURE 1: *Results of the examination for antibodies against Salmonella enteritidis using the FLOCKSCREEN® Salmonella enteritidis ELISA. Each point represents the result from one bird. The horizontal dash shows the arithmetic mean of each group. Comparison of antibody concentrations among chickens at week 24: G1 (Group 1) vs. G2 (Group 2) p = 0.0002, G2 vs. G3 (Group 3) p = 0.0002, G1 vs. G3 p = 0.007, at week 51: G1 vs. G2 p = 0.0014, G2 vs. G3 p = 0.0003, G1 vs. G3 p = 0.079, at week 71: G1 vs. G2 p=0.0199, G2 vs. G3 p = 0.0112, G1 vs. G3 p = 0.6626.*

group vaccinated three times with the live *S. enteritidis* vaccine (group 1) and the corresponding non vaccinated control group. In contrast a significant difference for the liver count between both groups was missed marginally (p = 0.066). To increase the statistical power further eight randomly selected birds from groups one and three were infected with the strain *S. typhimurium* 27 N in their 75th week of age and examined seven days post challenge. The results of the examination of the colonisation of liver and caecal mucosa with the *S. typhimurium* challenge strain were evaluated together with the results from the infection in the 73rd week (Tabl. 3). By increasing the number of birds it was also possible to confirm the difference for the liver between both groups with the appropriate statistical confidence (p < 0.05).

Discussion

The aim of the experiment was to examine the protective effects of vaccination with either *S. enteritidis* live vaccine Gallivac® Se or its combined use with Gallimune® Se+St inactivated vaccine against the serovars *S. enteritidis* und *S. typhimurium* over the entire laying period.

When evaluating the results of the quantitative examination of liver and caecal mucosa consideration has to be attributed to the challenge strain used, the challenge dose, the age of the animals and the time of the investigation (Methner, 1991). Even a primary infection with a virulent *S. enteritidis* strain and a challenge with a second virulent *S. enteritidis* strain does not lead to a complete reduction of the salmonella burden in the organs examined (Methner, 1991, Beal et al., 2006a). The reduction of the challenge strain content in liver and caeca in the present study complies with the investigations of other vaccine strains (Methner et al., 1995).

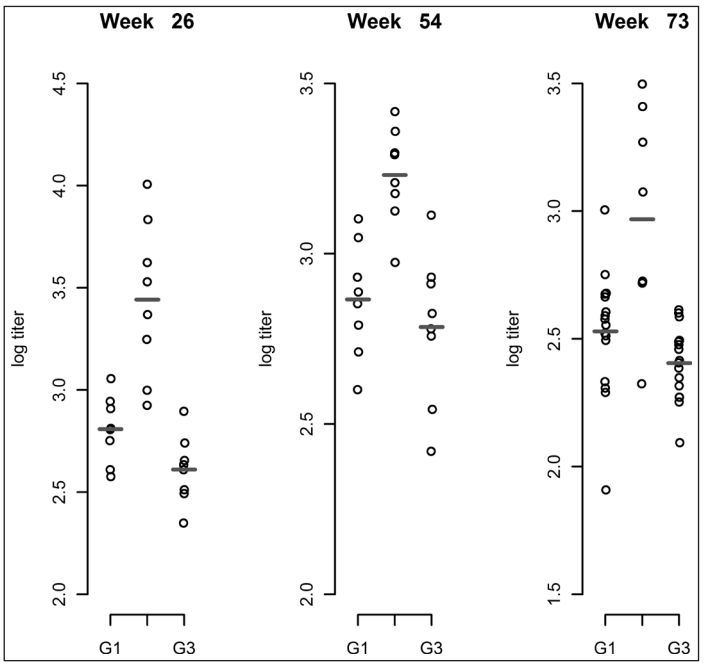

FIGURE 2: *Results of the examination for antibodies against Salmonella typhimurium using the FLOCKSCREEN® Salmonella typhimurium ELISA. Each point represents the result from one bird. The horizontal dash shows the arithmetic mean of each group. Comparison of antibody concentration among chickens at week 26: G1 vs. G2 p = 0.0011, G2 vs. G3 p = 0.0002, G1 vs. G3 p = 0.0406, at week 54: G1 vs.G2 p = 0.0006, G2 vs. G3 p = 0.0003, G1 vs. G3 p = 0.5235, at week 73: G1 vs. G2 p = 0.0045, G2 vs. G3 p = 0.0005, G1 vs. G3 p = 0.0267.*

TABLE 3: *Results of the challenge studies against Salmonella typhimurium (strain Salmonella typhimurium 27 N, measured seven days post challenge*

Group	n	Time of Challenge (Week of life)	Challenge strain content (mean ± SD) in log cfu/g Liver	Caeca
1	8	26	0.32 ± 0.60[1]	4.75 ± 0.72[1]
2	8	26	0.86 ± 0.57[1]	5.51 ± 1.14
3	8	26	1.84 ± 0.48	5.95 ± 1.01
1	8	54	0.85 ± 0.62[1]	5.85 ± 1.08
2	8	54	0.53 ± 0.83[1]	5.09 ± 0.58[1]
3	8	54	1.76 ± 0.33	6.14 ± 0.55
1	8	73	1.10 ± 0.53	5.78 ± 0.23[1]
2	8	73	0.81 ± 0.74[1]	5.59 ± 0.40[1]
3	8	73	1.71 ± 0.71	6.69 ± 1.04
1	16	73/75	0.96 ± 0.63[1]	5.82 ± 0.65[1]
3	16	73/75	1.74 ± 0.57	6.62 ± 0.97

Significant difference between the vaccinated group and the control group (Mann Whitney U test, one-tailed test)[1] significance level p < 0.05.

In conclusion both, the exclusive use of the live vaccine and its combined use with an inactivated vaccine in a vaccination programme were able to confer protective effects against *S. enteritidis* up to the end of the laying period, demonstrated by a significant reduction of the colonisation of the liver and caeca mucosa with the *S. enteritidis* challenge strain (147 N). This corresponds with the results from Atterbury et al. (2009) who showed a protective effect against *S. enteritidis* after vaccination with only the live vaccine (Gallivac® Se). At the same time the results confirmed the examinations from Hafez et al. (2001) who demonstrated a satisfactory efficacy for the combined use of live and inactivated vaccines in field trials. In addition to the homologous protective effect a cross immunity against *S. typhimurium* was shown after the *S. enteritidis* live vaccine had been administered three times. This also correlates with the results reported by Beal et al. (2006a) which demonstrated cross protection during challenge of chickens with a *Salmonella enteritidis* and re-challenge with a *S. typhimurium* field strain. On the other hand Cooper et al. (1994) demonstrated no cross protection against *S. typhimurium* after using an *aroA S. enteritidis* mutant (strain CVL 30) although satisfactory homologous protection was shown. The combined vaccination with the live *S. enteritidis* vaccine and the inactivated *S. enteritidis/typhimurium* vaccine demonstrated protection against *S. enteritidis* and protection against *S. typhimurium*.

The group vaccinated with both the live and the inactivated salmonella vaccines, showed significantly higher antibody concentrations against *S. enteritidis* and *S. typhimurium* before the challenge. The higher antibody concentrations of the birds in this group compared with the group vaccinated with the live vaccine for three times was not related to a higher reduction of challenge strain content in liver and caeca mucosa. These observations confirm the results from Beal et al. (2006b), Lehmann et al. (2006) and Carvajal et al. (2008), that the colonisation of organs with salmonellae is reduced primarily by cell mediated immune mechanisms.

In summary, the use of Salmonella vaccines should always be combined with other animal health measures including cleaning and disinfection, effective rodent control and biosecurity. In addition to this measures the triple use of the live vaccine Gallivac® Se or the combination of two doses of the live vaccine with one dose of

the inactivated vaccine Gallimune® Se+St prior to the laying period can be recommended as efficient measures to control *S. enteritidis* and *S. typhimurium* infections.

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